cDNA Cloning and Protein Analysis of a Bovine Dermal Allergen with Homology to Psoriasin

Jaakko Rautiainen, Marja Rytkönen, Sinikka Parkkinen, Jaana Pentikäinen, Annikka Linnala-Kankkunen,* Tuomas Virtanen, Jukka Pelkonen, and Rauno Mäntyjärvi

Departments of Clinical Microbiology and *Biochemistry and Biotechnology, University of Kuopio, Kuopio, Finland

Immunoscreening of a cDNA library from bovine skin led to isolation of clones coding for an allergen named BDA11. Sequence analysis of the clones revealed that they can encode a protein of 11.6 kDa with a predicted pI of 5.19. Allergenicity of BDA11 was verified by the IgE reactivity in cattle-allergic patients' sera with the recombinant protein produced in *Escherichia coli*. A biochemically purified native allergen of 11 kDa from bovine dander was identified as BDA11 by peptide sequencing. Homology comparisons showed that BDA11 had a 63.4% amino acid identity with human psoriasin. Psoriasin is a calcium-binding protein expressed in keratinocytes, and it is strongly up-regulated in psoriatic skin. BDA11 also had segments homologous with calcium-binding proteins from three other species. *J Invest Dermatol 105:660–663, 1995*

Primary structure of an allergen provides a basis for epitope mapping and for designing of more specific diagnostic reagents and more effective preparations for immunotherapy [1]. Peptide preparations of the house dust mite allergen Der p 1 containing T-cell epitopes induce non-responsivenes to immunogenic challenge both *in vitro* and in mouse experiments [2,3]. Similar results have been obtained with peptides from cat allergen Fel d 1 [4]. In addition to predicting potential epitope sites, primary structure can also be used to get information on the secondary structure and to build a three-dimensional model of the allergen [5]. Furthermore, a comparison with homologous proteins may lead to perception of the biologic activity of the allergen.

Cattle dander contains several allergens as shown by IgE immunoblotting with sera from cattle-allergic people [6]. The predominant bovine dander allergen is BDA20, which can be classified as a member of the lipocalin family (Mäntyjärvi *et al*, submitted). One of the minor allergens has been identified as the oligomycin sensitivity–conferring protein of the mitochondrial adenosine triphosphate synthase complex [7]. In the present paper, we describe the primary structure of another minor allergen, BDA11. The most interesting observation was the remarkable degree of homology between BDA11 and human psoriasin, a calcium-binding keratinocyte protein highly up-regulated in psoriatic skin.

MATERIALS AND METHODS

Preparation of mRNA and cDNA Cloning Total RNA was extracted from bovine skin as described previously [7]. Poly (A) RNA was purified with oligo dT-cellulose (Promega) and used for cDNA synthesis. cDNA was cloned into the Uni ZAP XR vector (Stratagene, ZAP-cDNA synthesis kit).

Screening of the cDNA Library The cDNA library was immunoscreened using pooled sera of patients with cattle allergy. Phage plaques were lifted onto isopropylthio-β-D-galactopyranoside-treated nitrocellulose filters. Filters were washed, blocked by 0.1% casein in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h and further incubated with diluted (1:5) patient serum for 6 h. ^{125}I-labeled anti-human IgE (Phadebas Rast, Pharmacia Diagnostics) was used as the secondary antibody with an overnight incubation. Filter washings and serum and conjugate dilutions were made in Tris-buffered saline with 0.05% Tween 20. After autoradiography, positive plaques were picked and purified with repeated phage dilutions and immunoblottings.

DNA hybridization was used to estimate the number of clones containing sequences homologous to isolated clones. Phage plaques were lifted onto nitrocellulose filters and hybridized according to a standard protocol [8] using probes prepared from individual isolated clones and labeled with [^{32}P]dCTP (Multiprime DNA-labeling kit, Amersham, England).

Sequencing of the cDNA Clones Inserts containing Bluescript SK(−) phagemids were excised from the bacteriophage Uni-ZAP XR lambda and transformed to *Escherichia coli*, XL1-Blue cells (Stratagene). DNA for sequencing was purified by Wizard Minipreps DNA Purification System (Promega). Nucleotide sequencing was done with the automatic DNA sequenator A.L.F., using an Auto Read kit (Pharmacia Biotech).

Expression Vector Construction and Recombinant Protein Expression Bluescript plasmid clone pB1 (from nucleotide 13 to the Kpn site in the vector, **Fig 1**) was first linearized at the 5′ end of the cDNA with EcoRI. The restriction site was then filled in with four dNTPs, and phosphorylated NcoI linker was ligated to this blunt end. The NcoI-KpnI fragment (438 bp) was excised from the DNA and was ligated into expression vector p*Trc*99A (Pharmacia, p*Trc*99A Expression Vector kit).

The recombinant plasmid p*Trc*99AB1-10 was transformed into *E. coli* host strain JM105 and selected on plates containing 100 μg/ml ampicillin. A single transformant colony was passed in Luria medium containing 100 μg/ml ampicillin. To produce the recombinant protein, 50 ml of prewarmed Luria medium was inoculated and grown at 37°C to A_{600} = 0.4. Protein expression was induced by the addition of isopropylthio-β-D-galactopyranoside to a final concentration of 2.5 mM. After 3 h growth, cells were harvested to 50 mM Tris-HCl buffer, pH 7.5, containing 220 mM NaCl, and lysed by freezing and rapid thawing. After centrifugation at 10,000 × *g* the supernatant that contained the solubilized proteins was stored at −20°C.

Immunoblot Analysis The freeze-dried material from bovine dander was purchased from Allergon AB (Ängelholm, Sweden). The material was

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Sinikka Parkkinen's present address: Department of Chemical Technology, Lappeenranta University of Technology, Lappeenranta, Finland.

Reprint requests to: Dr. Rauno Mäntyjärvi, Department of Clinical Microbiology, University of Kuopio, P.O.B. 1627, 70211 Finland.

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1          10         20         30         40         50
GTGACATCTCCTCTGATCAGCTCTTTGGAAGCCAAGATGAGCAGCTCTCAGCTTGAGCAG
                                       M  S  S  S  Q  L  E  Q

61         70         80         90         100        110
GCCATTACAGACTTGATCAACCTGTTTCACAAATACTCGGGATCCGATGACACCATCGAG
 A  I  T  D  L  I  N  L  F  H  K  Y  S  G  S  D  D  T  I  E

121        130        140        150        160        170
AAGGAGGACCTGCTGCGGCTGATGAAGGACAACTTCCCCAACTTCCTCGGTGCCTGTGAG
 K  E  D  L  L  R  L  M  K  D  N  F  P  N  F  L  G  A  C  E
                            E*

181        190        200        210        220        230
AAAAGGGGCAGAGATTACTTGTCCAATATCTTTGAGAAACAAGACAAGAATAAGGACCGG
 K  R  G  R  D  Y  L  S  N  I  F  E  K  Q  D  K  N  K  D  R

241        250        260        270        280        290
AAGATTGACTTTTCTGAGTTCCTGTCCTTGCTGGCGGACATAGCCACAGACTATCACAAC
 K  I  D  F  S  E  F  L  S  L  L  A  D  I  A  T  D  Y  H  N

301        310        320        330        340        350
CACAGCCACGGAGCACAGCTCTGTTCTGGGGGAAATCAGTGAGCCCAGAGGCCTCCGGAC
 H  S  H  G  A  Q  L  C  S  G  G  N  Q

361        370        380        390
AACCCAAGAAACAATAAAGTGTCTTTTCTCACCAGAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide and amino acid sequences of the BDA11 allergen. The sequence of BDA11 (GSDB accession number L39834) was compiled from the sequencing results of three clones. *Underlined* amino acid sequences were verified by micro sequencing of the native BDA11 [10]. There was one difference (*) between the deduced and observed amino acid sequence.

defatted in diethyl ether (1:10 w/v) at 4°C for 24 h and then extracted for 24 h at room temperature in Coca's solution, pH 7.2 (1:10 w/v; 0.5% sodium chloride, 0.275% sodium hydrogen carbonate, 0.4% phenol). After centrifugation, the supernatants were dialyzed (molecular weight cutoff 3500) against distilled water, lyophilized, and stored at −20°C. Immunoblottings of the bovine dander extract and recombinant proteins were carried out as described previously [6]. Briefly, proteins were first separated in sodium dodecylsulfate–polyacrylamide gel electrophoresis (12.5% gel) using the discontinuous buffer system and transferred to a nitrocellulose membrane. The membrane was then blocked in phosphate-buffered saline, pH 7.3, containing 0.1% casein, 0.05% Tween 20, and 0.05 w/v NaN_3 for 1 h at 37°C and cut into strips. The strips were incubated overnight at room temperature with serum from a cattle-asthmatic person (diluted 1:5). The strips were then washed in Tween-PBS four times for 5 min and incubated overnight with ^{125}I-labeled anti-human IgE (Phadebas RAST) or with rabbit anti-mouse Ig followed by ^{125}I-labeled donkey anti-rabbit Ig antiserum. After washing, the reactive bands were visualized with autoradiography.

Affinity Purification of the IgE Serum was adsorbed to the recombinant protein as described by Donovan and Baldo [9]. Phage plaques were plated at a high density on a plate of 100-mm diameter. Synthesis of the fusion protein was induced by an isopropylthio-β-D-galactopyranoside–treated nitrocellulose filter. The filter was lifted and washed briefly with Tris-buffered saline with 0.05% of Tween 20. Proteins were fixed onto the filter by 0.5% glutaraldehyde in phosphate-buffered saline and blotted with a serum of a cattle-allergic patient, diluted 1:4 in Tris-buffered saline with 0.1% casein. Antibodies were eluted with 0.1 M glycine-HCl, pH 3.0, neutralized by 1 M Tris, pH 9.5, concentrated by ultrafiltration (Centricon 3, Amicon, molecular weight cutoff 3000), and used for immunoblotting.

Proteolytic Digestion and Amino Acid Sequence Analysis Lyophilized bovine dander extract was dissolved into phosphate-buffered saline. The extract was fractionated by gel filtration through an XK 16/70, Superdex 75 column using the HiLoad Pump P-50 (Pharmacia Biotech). The elution was monitored with Uvicord III (LKB), and 40 2-ml fractions were collected. The protein contents of fractions were examined with sodium dodecylsulfate–polyacrylamide gel electrophoresis.

The 11-kD protein was found in gel-filtration fractions corresponding to a molecular weight of about 22 kD (Rautiainen *et al*, submitted). One of these fractions was concentrated by ultrafiltration (Centricon 3, Amicon). Sodium dodecylsulfate–polyacrylamide gel electrophoresis of the sample was carried out in a 15% acrylamide gel using the discontinuous buffer system. The gels were stained with Coomassie brilliant blue and destained with 30% methanol.

In-gel protease digestion for amino acid sequencing of peptides was done with minor modifications as described by Rosenfeld *et al* [10]. The 11-kD protein bands from six lanes of a gel were cut out, pooled, and washed three times with 50% acetonitrile-ammoniumbicarbonate buffer (100 mM, pH 8.9) at 30°C for 20 min. Dried gel slices were swollen partly with 10 μl of 200 mM ammoniumbicarbonate buffer containing 0.02% Tween 20, pH 8.9, and 4 μg trypsin (Boehringer Mannheim) in 8 μl of 0.01% trifluoroacetic acid (TFA) was added. Ammoniumbicarbonate buffer was added slowly to cover the gel slices, and the incubation was carried out overnight. The enzyme digestion was stopped with 1.5 μl of 100% TFA and the peptides were extracted in a shaker twice with 60% acetonitrile–0.1% TFA and once with the same solution containing 0.02% Tween 20. The digestion solution and the extracts were pooled together and lyophilized.

The peptides generated by trypsin treatment were separated by high-performance liquid chromatography (Vydac C-18 column) with a linear acetonitrile gradient (0–60% acetonitrile in 0.1% trifluoroacetate in water) and lyophilized. Applied Biosystems 477A Pulsed Liquid Phase protein sequencer was used to determine the amino acid sequences of five well-resolved peptides.

Homology Comparisons and Epitope Predictions GCG program package was used for homology comparisons [11]. The analysis for possible T-cell epitope sequences was performed with the TSites program [12] for a Macintosh computer.

RESULTS

Sequencing and Expression of cDNA A cDNA library from bovine skin was screened with IgE immunoblotting by using a pool of sera from cattle-allergic patients. Several positive clones were isolated and the inserts were sequenced. Three of the clones contained inserts of various lengths with the same sequence. The EcoRI-KpnI insert of 427 bp was excised from the clone pB1 and used as a probe to screen the original cDNA library. The prevalence of plaques reacting with this probe was 0.24%. Sequencing results of the three clones are compiled in **Fig 1**. The longest open-reading frame has a methionine codon at nucleotide 37. The protein from this methionine to the stop codon at nucleotide 340 has the size of 101 amino acids, a molecular weight of 11.55 kDa, and a predicted isoelectric point of 5.19.

To verify allergenicity of the protein, insert of the cDNA clone pB1 was recloned in frame into the Trc expression vector. As shown in **Fig 2**, the recombinant protein expressed in *E. coli* bound IgE antibodies from the serum of a pätient with cattle allergy. Next, the recombinant protein expressed in phage plaques was used to adsorb IgE antibodies from a patient's serum. When the adsorbed and eluted antibodies were used to stain allergens in bovine dermal extract by immunoblotting, a positive signal was seen corresponding to an 11-kDa protein **(Fig 3)**. This allergen was tentatively named BDA11. When the reactivity of sera from a group of patients with verified cattle-associated asthma was tested against the recombinant BDA11 protein by IgE immunoblotting, seven of 16 were positive. In comparison, sera of nine symptomless dairy farmers were negative.

Microsequencing of tryptic peptides of BDA11 purified from bovine dander by gel filtration and sodium dodecylsulfate–polyacrylamide gel electrophoresis was used to link native BDA11 to the cDNA clones. Repeated sequencing of several peptides separated by high-performance liquid chromatography resulted in amino acid sequences that were identical with one exception (at nucleotide 149) to those encoded by the cDNA (underlined in **Fig 1**).

Homology Comparisons A search in the gene libraries with BDA11 protein and cDNA sequences revealed a 63.4% identity of amino acids between BDA11 and human psoriasin **(Fig 4)**. In the C-terminal half there is a segment where 18 of 19 amino acids are identical. Psoriasin is one of the calcium-binding proteins, and the calcium-binding motif containing the so-called EF hand is located in psoriasin in the same segment as the highest homology with BDA11. Another search was made with sequential segments of 33 amino acids of BDA11. Whereas the middle and C-terminal segment only brought up psoriasin, the N-terminal segment showed a remarkable similarity to a group of other calcium-binding proteins in addition to psoriasin. The homology comparison between the

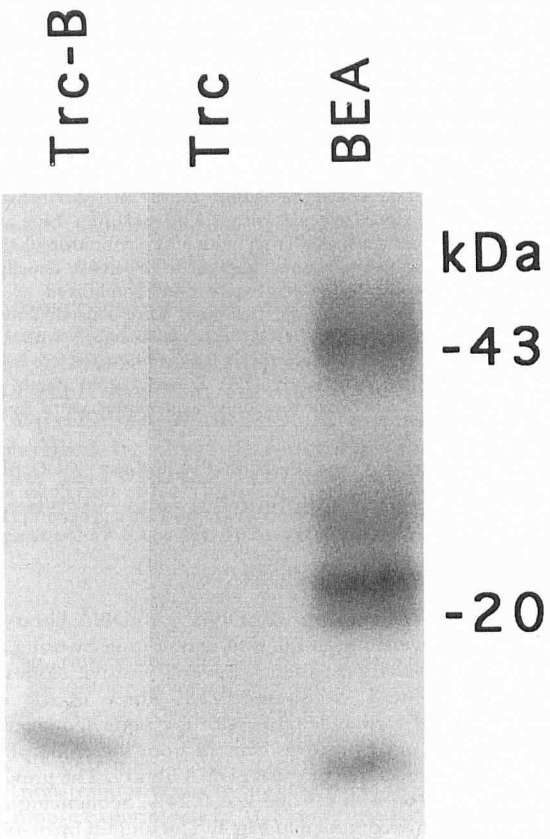

Figure 2. Human IgE antibody reacts against recombinant BDA11 protein. Serum from a cattle-allergic patient was used in immunoblotting against bovine epidermal allergen extract (BEA), rBDA11 from Trc vector (Trc-B), and Trc vector without the allergen insert (Trc).

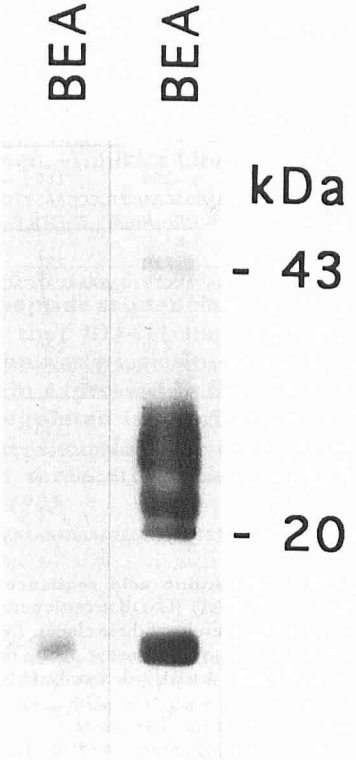

Figure 3. Affinity-purified anti-BDA11 IgE antibodies react against bovine epidermal allergen extract (BEA). Untreated serum (*right lane*) or affinity-purified anti-BDA11 IgE antibodies (*left lane*) from a cattle-allergic patient were used in immunoblotting against bovine epidermal allergen extract (BEA).

N-terminal third of BDA11, psoriasin, and four other calcium-binding proteins is shown in **Fig 5**. Three blocks of homologous amino acid sequences confirmed the close relationship between BDA11 and proteins from four different species.

Secondary Structure and Possible Epitopes of BDA11 Peptide sequences suggested as possible T-cell epitopes by the Tsites program's two algorithms are marked in **Fig 4**. Both algorithms proposed six partially overlapping segments as T-cell epitope sites. Two of the sites are located at the C-terminal, highly conserved third of BDA11.

DISCUSSION

We describe in this paper cDNA and amino acid sequences of a new mammalian dander allergen, BDA11. BDA11 can be classified as a minor allergen by the IgE antibody prevalence among cattle-allergic patients, but the high number of phage plaques encoding BDA11 detected in the cDNA library by the DNA hybridization screening suggests that it is present in significant amounts in bovine skin.

The most interesting observation in the present study was the remarkable degree of homology between BDA11 and human psoriasin. The latter is a protein found in human keratinocytes [13] and related to a subgroup of calcium-binding proteins [14]. It can be found in normal human skin and in some other tissues but it is highly up-regulated in psoriatic skin. The function of psoriasin is unknown but its up-regulation is apparently associated with inflammation and abnormal proliferation of keratinocytes in the psoriatic lesion [14]. It is tempting to speculate that BDA11 is in fact the bovine equivalent of psoriasin. The amino acid sequence homology of BDA11 is markedly higher to psoriasin than to any other protein identified so far. The highly homologous region containing the calcium-binding motif is identical in both proteins, compatible with the same conserved function in keratinocytes. The presence of homologous blocks of amino acids in the N-terminal third of BDA11 and calcium-binding proteins from four other species further indicates an evolutionary and functional relationship. Although the function of BDA11 and human proriasin in keratinocytes may be identical their expression in normal skin is different. Psoriasin can be found in high amounts only in psoriatic lesions whereas BDA11 seems to be abundant in normal bovine skin. It is too early to speculate to what degree the normal bovine skin and a psoriatic lesion are comparable.

Biologic functions of mammalian allergens are largely unknown. We have earlier identified one of the minor allergens in bovine

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                .         .         .         .         .
  1 MSNTQAERSIIGMIDMFHKYTRRDDKIDKPSLLTMMKENFPNFLSACDKK 50
    ||..| |..|.::|::|||||. .||.|:|..|| :||:||||||:||:|:
  1 MSSSQLEQAITDLINLFHKYSGSDDTIEKEDLLRLMKDNFPNFLGACEKR 50
    ........**********.............***....*******....
    ..........++++.........++++..+++++++.............
                .         .         .         .         .
 51 GTNYLADVFEKKDKNEDKKIDFSEFLSLLGDIATDYHKQSHGAAPCSGGSQ 101
    | :||.::|||.|||.|:|||||||||||:|||||||.:|||||. ||||.|
 51 GRDYLSNIFEKQDKNKDRKIDFSEFLSLLADIATDYHNHSHGAQLCSGGNQ 101
    ....*****.............**********..*****.............
    ..++++.................++++...+++++.................
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Figure 4. Homologous segments of amino acid sequences are found in human psoriasin (*upper line*) and BDA11 (*lower line*), and predicted T-cell epitopes are localized in BDA11. T-cell epitope predictions were done by using two different algorithms [12]. *AMPHI and (+) Rothbard/Taylor algorithms.

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bda11_1-33  .mss SQLE q A itdLinl FHKY s G sdd...tie KEDL l..
psor_human  .msn tQaE r s iigMidm FHKY t r rdd...kid KpsL l..
cl1l_xenla mvap SeLE h s mekMllt FHKF e G ekn...ymn rDDL ...
cl1l_chick  ...p SQME h A metLmft FHKY a G dkn...yls KEDL ral
cl1l_mouse  ...p SQME h A metMmlt FHrF a G dkd...hlt KEDL rvl
s10b_bovin  .... SeLe k A vvaLidv FHqY s G rdkhklkks elk. ..

Consensus   ---- SQLE - A ------- FHKY - G --------- KEDL---
```

Figure 5. Homologous regions are identified in BDA11 and four calcium binding proteins. Aligning and consensus sequence search [11] of BDA11 amino acids 1 to 33, human psoriasin (psor_human), cl1l protein of African clawed frog (cl1l_xenla), cl1l protein of chicken (cl1l_chick), cl1l protein of mouse (cl1l_mouse), and s10b protein of cow (s10b_bovin). Homologous segments are enclosed in boxes.

dander as oligomycin sensitivity conferring protein of the mitochondrial adenosine triphosphate synthase complex [7]. The predominant allergen in bovine dander is a member of the lipocalin family [Mäntyjärvi *et al*, submitted], which are proteins carrying lipophilic molecules. Lipocalins also include urinary allergens of rodents [15,16]. The present study adds a keratinocyte protein on the list of allergens. It is apparent that there is no biologic function common to allergenic mammalian proteins. In contrast, many of the allergens from mites and bees have been identified as proteinases. The major allergens of three species of mites, Der p 1, Der f 1, and Eur m 1 have all shown cysteine protease activity [17–19]. Serine-protease activity was associated with Der p 3 and amylase activity with Der p 4 [20,21].

The homology between BDA11 and human psoriasin raises the question of immunogenic/allergenic properties of BDA11. Mapping of both B-cell (IgE-reactive) and T-cell epitopes of BDA11 with the help of antibodies and T-cell clones from allergic persons is essential for assessing the significance of the homology between BDA11 and psoriasin. Two of the predicted T-cell epitopes in BDA11 are within the conserved calcium-binding motif. It would be logical that the reactive epitopes were found in the non-homologous region but autoreactivity has been suggested as a possible mechanism in the pathogenesis of allergic reactions [22]. If autoreactivity existed it would be expected to have a role in cattle-associated allergic dermatitis, which is also a well known work-related disorder in dairy farmers [23].

Expression of a conserved allergenic protein in bovine skin also raises the question of allergenic properties of related proteins in dander of other mammalian species. An example of a similar situation is offered by profilins. They are a group of conserved actin-binding proteins present in eukaryotic cells and possessing cross-reactive allergenic properties [22]. Autoreactive T-cell clones in individuals sensitized to BDA11 or a cross-reactive protein from other species could respond against psoriasin when it is up-regulated in the skin by injury or infection, both predisposing factors of psoriasis. Those genetically predisposed to produce T-cell responses with high levels of psoriasis-associated cytokines interleukins 2, 4, and 6 and interferon γ [24] would have a long-lasting inflammation leading to a psoriatic lesion, the others only a short-lived reaction. We are currently exploring the possibility that the minor dander allergens of other mammals might include BDA11-related proteins.

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